

Pattern of IGF-1 Variants' Expression in Human Cancer Cell Lines Using a Novel q-RT-PCR Approach

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Abstract. *Background/Aim:* Although insulin-like growth factor-1 (IGF-1) is well-implicated in cancer biology, the potential roles of IGF-1 variants in different types of cancer are largely unknown. The aim of the present study was to *in vitro* characterize several human cancers for their IGF-1 variant expression patterns. *Materials and Methods:* Using a novel quantitative real-time polymerase chain reaction (qRT-PCR) assay, twelve human cancer cell lines were investigated for their endogenous expression levels of IGF-1 variants, including classes. Additionally, the hormonal regulation of IGF-1 transcripts was investigated in PC3 cells. *Results:* IGF-1Ea and Eb were found at higher levels in KLE and MEL28 cells, respectively. MCF7 had the lowest expression of Ec peptide and, along with MB231, lacked IGF-1Eb. In most cases, class 1 proved as the predominant origin. Estradiol (E2) or dexamethasone (Dexa) significantly modulated IGF-1Ea and IGF-1Eb and down-regulated the Ec peptide in PC3. *Conclusion:* Our results contribute to the notion of distinct roles of IGF-1 isoforms in human cancer depending on the type of malignancy.

The insulin-like growth factor-1 (IGF-1) is a potent mitogen of major importance in human physiology and development with numerous proliferative and anti-apoptotic effects addressed (1). The 100kb *IGF-1* gene is located on the long arm of chromosome 12 (*12q22-12q24*) and it consists of 6 exons (2, 3). Combination of different transcription initiation sites, alternative splicing at both 5' and 3' ends and post-translational modifications, such as proteolytic processing and glycosylation events, give rise to multiple products

(isoforms) (4). More specifically, the precursor IGF-1 polypeptides consist of the signal peptide, mature IGF-1 and the E peptides; mature IGF-1 is a single-chain 70-amino-acid (aa)-peptide consisting of exons 3 and 4, while at the carboxyl (C)-terminal, extension of the pro-IGF-1 peptides and combination of exons 5 and 6 results in distinct E peptides, named Ea, Eb and Ec. Thus, alternative splicing, containing only exon 5, results in the IGF-1Eb isoform, whereas containing exon 6 but excluding exon 5 results in IGF-1Ea (5). A third variant, the IGF-1Ec, is also generated by alternative splicing in the human *IGF-1* gene and contains both exons 5 and 6 (6). At the N-terminal, usage of different promoters, located either in exon 1 or 2 produces class 1 or class 2 IGF-1 molecules, respectively. Pro-IGF-1 peptides consist of mature and E peptides (4) (Figure 1A).

Although over the last decade an increasing number of studies indicate a differential association of IGF-1 isoforms with several pathophysiological processes, such as myocardium remodeling, skeletal muscle regeneration and endometriosis (7-9), the potentially distinct molecular functions of IGF-1 E peptides, and especially of its different classes, are largely unknown. Interestingly, over the last years, focus has turned into clarifying the role of specific IGF-1 isoforms in cancer biology (9-12). The differential expression pattern of IGF-1 isoforms observed in *in vitro* models, indicate a unique and distinct role of pro-peptides in different cancer types including osteosarcoma, prostate, endometrial and cervical malignancies (10-12). However, less is known about the possibly specific roles of the IGF-1 isoforms or, particularly, of its different classes in the various types of cancer.

Against this background, the aim of the present study was to characterize several immortalized cell lines, the majority of which served as models of numerous human cancer types, regarding their expression pattern of IGF-1 isoforms at the mRNA level, including their classes, under control and/or hormonal conditions. Twelve cell lines were used simulating, among others, breast, hepatocellular, lung, melanoma and colon malignancies. The results presented herein indicate for the first time the distinct identification of IGF-1 transcripts in so many

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Table I. Sequences of oligonucleotides used for amplification and quantification of the distinct IGF-1 variants in the qRT-PCR assay and the resulting products' length.

IGF-1 variant	Primer pair (exon location and DNA strand)	Sequences (5'-3')	Product length (bp)
Ea	IGF-1 exon 4 sense	GTATTGCGCACCCCTCAAG	94
	IGF-1 Ea exon 4-6 anti-sense	CAAATGTACTTCCCTTCTGGGTC	94
Eb	IGF-1 exon 4 sense	GTATTGCGCACCCCTCAAG	234
	IGF-1 Eb exon 5 anti-sense	CTACTTCCAATCTCCCTCCTCTG	234
Ec peptide	Ec peptide exon 5 sense	GTTTCTTATCAGCCCCATCTACCA	87
	Ec peptide exon 6 anti-sense	GTTTCTTACTTGCCTTCTTCAAATGTAC	87
Class 1 Ea	Class 1 exon 1 sense	ATCAGCAGTCTTCCAACCCA	405
	IGF-1 Ea exon 4-6 anti-sense	CAAATGTACTTCCCTTCTGGGTC	405
Class 1 Eb	Class 1 exon 1 sense	ATCAGCAGTCTTCCAACCCA	545
	IGF-1 Eb exon 5 anti-sense	CTACTTCCAATCTCCCTCCTCTG	545
Class 1 Ec peptide	Class 1 exon 1 sense	ATCAGCAGTCTTCCAACCCA	468
	Ec peptide exon 6 anti-sense	CTACTTGCCTTCTTCAAATGTAC	468
Class 2 Ea	Class 2 exon 2 sense	ATGATTACACCTACAGTGAAGATG	366
	IGF-1 Ea exon 4-6 anti-sense	CAAATGTACTTCCCTTCTGGGTC	366
Class 2 Eb	Class 2 exon 2 sense	ATGATTACACCTACAGTGAAGATG	506
	IGF-1 Eb exon 5 anti-sense	CTACTTCCAATCTCCCTCCTCTG	506
Class 2 Ec peptide	Class 2 exon 2 sense	ATGATTACACCTACAGTGAAGATG	429
	Ec peptide exon 6 anti-sense	CTACTTGCCTTCTTCAAATGTAC	429

IGF-1, Insulin-like growth factor-1; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

malignant tissue culture models, further supporting the notion for differential and unique roles of IGF-1 isoforms in human cancer biology, depending on the type of malignancy.

Materials and Methods

Primer design. All primer sets designed *de novo*, for increased efficiency and specificity, to be complementary to the unique exons corresponding to each IGF-1 transcript (Figure 1A) and within the coding region using the sequencing provided by the National Center for Biotechnology Information (NCBI), while their specificity was checked using the NCBI Primer BLAST tool. Forward primers for pro- and class 1 or 2 IGF-1 peptides were common for all primer sets and located to exon 4 and 1 or 2-3 (junction) exons, respectively, while reverse primers for pro-IGF1-Ea and -Eb were located to exons 4-6 (junction) and 5, respectively. Amplification of the IGF-1Ec transcript corresponding to the last (C-terminal) 24 aa of bioactive protein (Ec peptide) was achieved using a primer's pair complementary to exon 5 (sense) and exon 6 (antisense) (Table I, Figure 1A). Efficiency of all primer's sets was validated before tested to cells using serial dilutions in cDNA obtained from human muscle tissue, which served as positive control. All sets were found to have acceptable efficiencies, between 90 and 120% (Figure 2A), and unique curve picks in predicted for their length and GC content melting temperature, as shown in melting curve analysis (Figure 3A-I) in the same cycling conditions. Both sequencing (not shown) and electrophoretic analysis of the real-time PCR products in agarose gel (*e.g.*, Ec peptide is shown, in Figure 2B) further verified the specificity of each target transcript. Although all primer sets were designed to correspond to intron-exon spanning regions or to exon-exon junctions (Figure 1A and B), they were checked for their contaminating DNA amplification potential in a boosted PCR

reaction of 45 cycles using 300 ng of genomic DNA as template. The resulting PCR product was further analyzed in agarose gel electrophoresis where no bands were observed (Figure 2C). Primers designed for amplification of genomic sequence of cyclophilin were used as positive control of the reaction.

Cell cultures and treatments. The following employed normal and cancerous human cell lines were obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA): normal lens epithelial (HLE-B3), androgen sensitive prostate adenocarcinoma (LnCaP), androgen negative prostate cancer (PC3), estrogen positive breast adenocarcinoma (MCF7), estrogen negative breast adenocarcinoma (MDA-MB-231), hepatocellular carcinoma (HuH7), normal kidney (HEK293), endometrial cancer (KLE), epithelial cervical cancer (HeLa), lung cancer (A549), melanoma (SK-MEL28), osteosarcoma (MG63) and colon cancer cells (DLD1). All cell lines were cultured in selective growth medium supplemented with 10-20% fetal bovine serum (FBS) and/or 2mM L-glutamine (Lonza, Basel, Switzerland) according to ATCC instructions and 1% penicillin/streptomycin (GIBCO, Life Technologies, Grand Island, NY, USA) and maintained in an tissue culture incubator at 37o C and 5% CO₂. At control conditions, cells reached confluence of approximately 80% before harvesting. Adherent cells were washed twice with phosphate buffer salina (PBS) prior to trypsinization (GIBCO). Estradiol (E2), Dexamethasone (Dexa) and Growth Hormone (GH) (Sigma-Aldrich, St. Louis, MO) were selected as treatment factors and used in concentrations of 1, 10, 25 and 100 nM for 24 and 48 h in a dose- and time-response manner respectively.

RNA extraction and cDNA synthesis. Total RNA was extracted from cells using the TRI reagent RT-111 (RT-111; MRC Inc., Cincinnati, OH, USA) according to manufacturer's instructions. RNA concentration was photometrically measured using the optical density

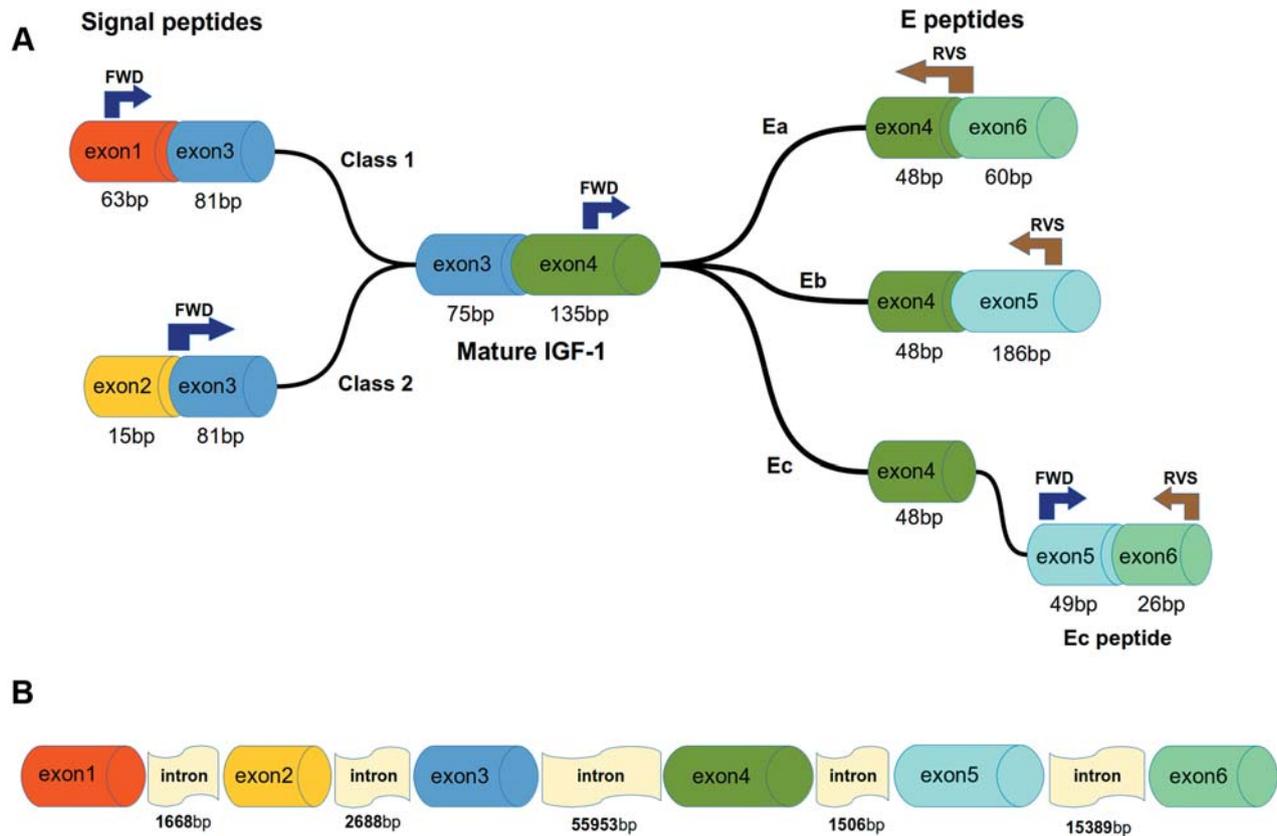


Figure 1. Schematic representation of IGF-1 alternative splicing peptides. Mature IGF-1 consists of exons 3 and 4. At the N-terminal the signal peptides consist of exon 1 or 2 spliced with part of exon 3 and give rise to class 1 or 2 molecules, respectively, whereas at the C-terminal combinations of exon 4 with exon 5 or 6 or both produce the E peptides named Ea, Eb and Ec. Primers used for amplification of alternative IGF-1 transcripts are marked as arrows and are located in their designed complementary exon or exon-exon junction. Forward (sense) primers are presented as right (clockwise) blue arrows, whereas reverse (antisense) primers as left (reverse clockwise) brown arrows (A). The intron-exon spanning or exon-exon junction design of primers and the length of intervening introns (B) eliminates the possibilities of contaminating DNA amplification. IGF-1, Insulin-like growth factor-1.

absorbance at 260 nm in a nano-drop spectrophotometer, whereas the absorbance ratio at 260/280 nm and the identification of 18s and 28s bands in 1% agarose gels after RNA electrophoresis were used to evaluate the quality of extracted RNAs (not shown). Following DNase treatment (Invitrogen, Life Technologies, Grand Island, NY, USA) one microgram (1 μ g) of each RNA were processed to cDNA synthesis using 0.5 μ g oligo DT primers (Fermentas, GmbH, Germany), 0.5 mM dNTPs (HT Biotechnology, Cambridge, UK), 200 U of M-MuLV reverse transcriptase (Finnzymes, Thermo Fisher Scientific, Pittsburgh PA, USA) and 4 U ribonuclease inhibitor (Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

Quantitative reverse transcription real-time PCR. Equal amounts (20 ng) of the obtained cDNAs were further quantified for the expression of IGF-1 isoforms using 0.4 μ M of each primer set described above and 1x Kapa SYBR FAST qPCR kit (KAPA Biosystems, Wilmington, MA, USA) in the same cycling conditions (95°C for 30 s, 95°C for 3 s, 63°C for 30 s, for 40 cycles) for all transcripts tested. The levels of the different variants of transcripts

were assessed by calculating the threshold cycle (Ct) as the number of cycles at which the measured fluorescence exceeded the threshold for detection, which was automatically set by the Bio-Rad iQ5 Real-Time PCR Detection System (Hercules, CA, USA) software. Each sample was analyzed in triplicate and the resulting data were averaged. A melting curve (Tm) analysis was also generated by the system's software following the final cycle (40) for each experimental sample by continuous monitoring the SYBR green fluorescence throughout the temperature ramp from 65° to 95°C. QPCR products were verified after both melting curve analysis and agarose gel electrophoresis prior to relative quantification. Relative differences between IGF-1 variant expressions after hormonal treatments and control conditions in PC3 cells were estimated as fold changes using the ddCT method. Differences in expression levels of IGF-1 isoforms within cell lines used were performed by comparing the mean \pm standard deviation (SD) of individual difference between threshold cycle values (dCT). In all cases β -actin (FWD: CCTCGCCTTTGCCGA , RVS: TGGTGCCTGGGGCG) served as an internal control.

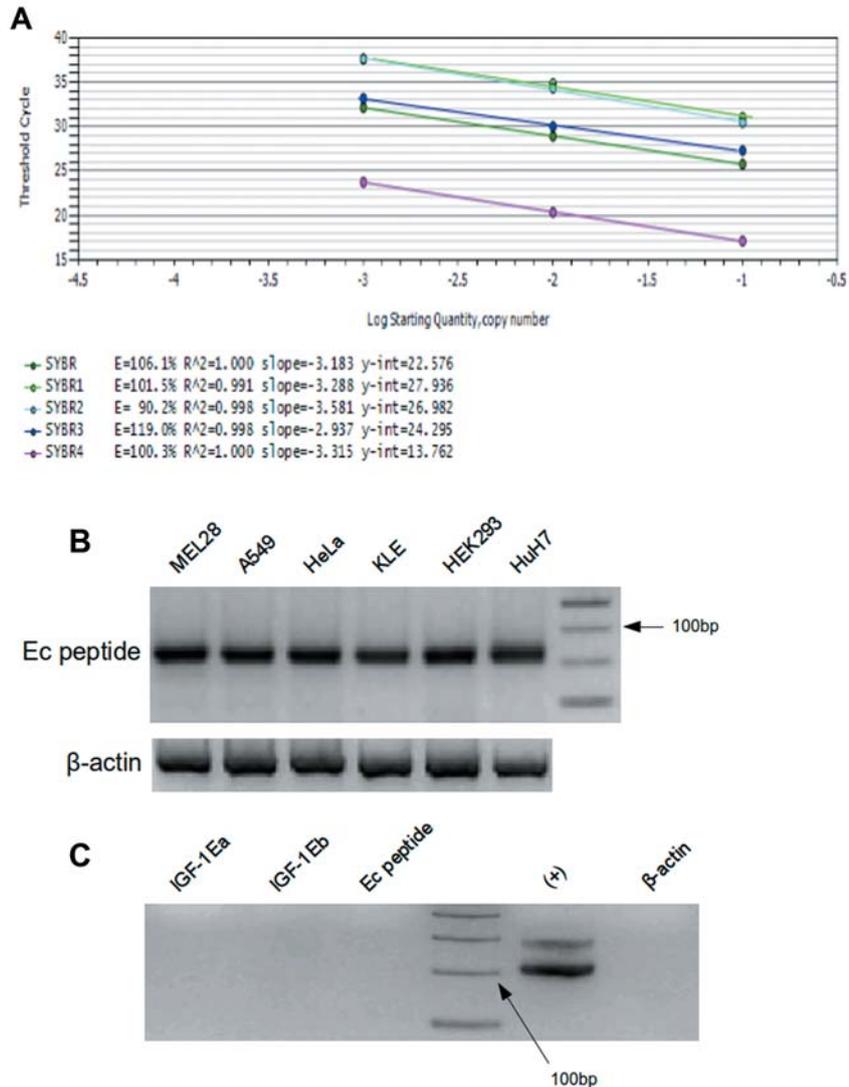


Figure 2. Efficiencies and specificity of primers used for quantification of IGF-1 variants. Efficiency of primers' amplification used in the qRT-PCR assay was checked using serial dilutions of cDNA obtained from human muscle tissue and was calculated from the slopes of standard curves. All primer sets were found to have acceptable efficiencies between 90% and 120%, e.g., calculated efficiencies for Class 1 Ea and Eb correspond to percentages of 106 and 101 (SYBR and SYBR1), whereas for IGF-1Ea, IGF-1Eb and Ec peptide to 90, 119 and 100, respectively (SYBR2-4) (A). Following melting curve analysis, qRT-PCR products (e.g., Ec peptide) were further verified by gel electrophoresis, where only one band was observed corresponding to the predicted length (B). The possibility of potentially contaminating DNA amplification was checked in a boosted PCR of 45 cycles using the designed primers (e.g., IGF-1Ea, IGF-1Eb, Ec peptide) or the primers used for amplification of β -actin, which served as internal control for transcripts quantification and 300 ng of genomic DNA as a template. Products were analyzed electrophoretically, where no bands were observed, using as positive control cyclophilin. (C). qRT-PCR: Quantitative real-time-polymerase chain reaction.

Statistical analysis. Unpaired *t*-test and/or regression analysis were used for the fold changes or the dCTs values given and a *p*-value <0.05 was considered as significant.

Results

Identification of distinct IGF-1 isoform expression patterns unique for each cell line under control conditions. Cell lines were cultured under their individual suggested control

conditions and examined for their mRNA levels of IGF-1 isoforms as described in Materials and Methods. The characterization of several human cancer cell lines concerning the expression pattern of IGF-1 Pro- and E-peptides is shown in Figure 4A. More specifically, the KLE and SK-MEL28 cell lines were found to have the maximum expression of pro-IGF1-Ea and -Eb peptides, respectively. Additionally, KLE and MEL28 cells, along with DLD1 and

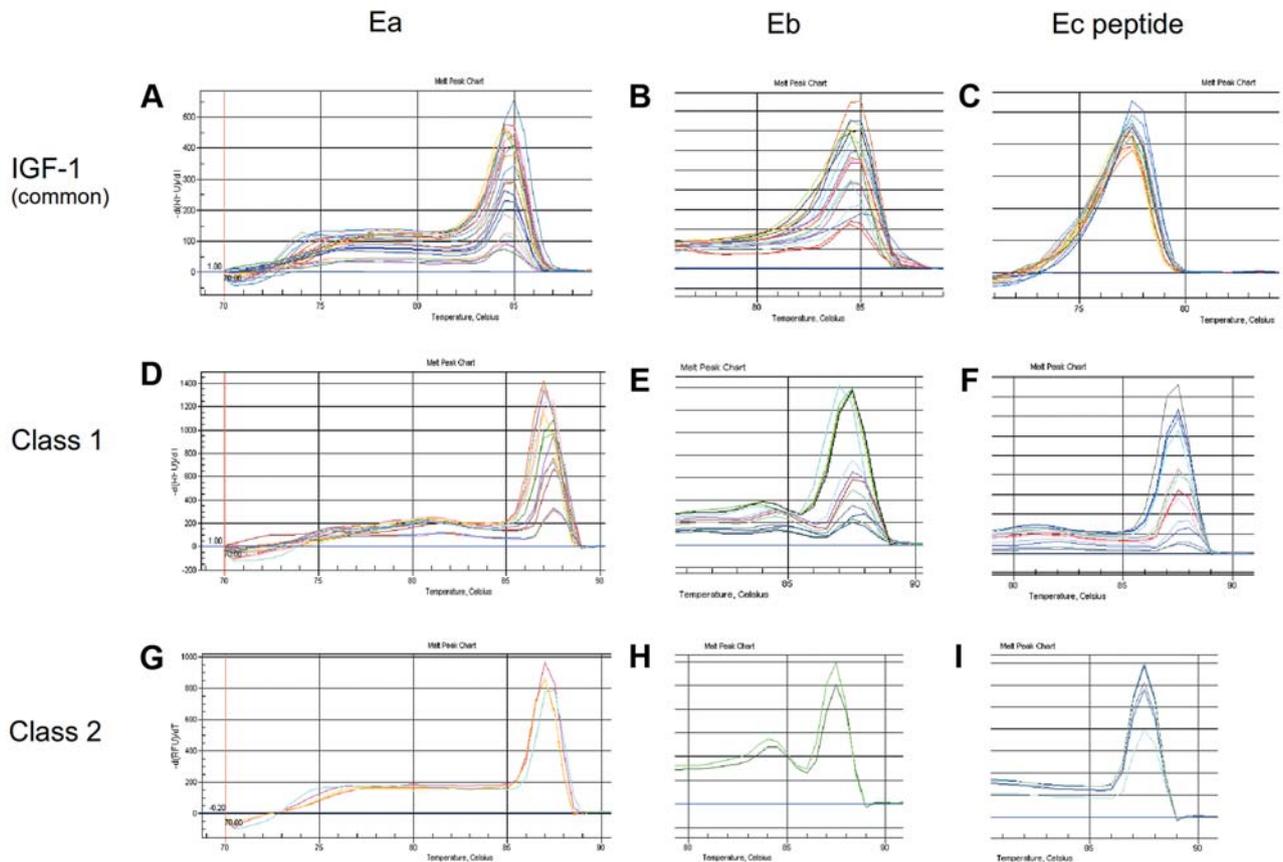


Figure 3. The melting curve analysis of the distinct IGF-1 transcripts. Following qRT-PCR's last cycle (40), a melting curve analysis was generated by the system's software measuring SYBR green fluorescence throughout a temperature ramp from 65°C to 95°C. Analysis showed a unique curve pick in specific for each transcript temperature, indicating the existence of a single product in each qRT-PCR reaction either for IGF-1Ea and IGF-1Eb isoforms (A, B) or Ec peptide (C), as well as for class 1 Ea, Eb and Ec peptide molecules (D-F) or class 2 Ea, Eb and Ec peptide molecules (G-I). qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; IGF-1, insulin-like growth factor-1.

LnCap, were found to have elevated levels of both IGF-1Ea and IGF-1Eb isoforms, compared to others. The Ec peptide was also found at maximum levels in KLE cells, with LnCap following, whereas MCF7 cells presented the lowest expression compared to the rest of tested cell lines. All three isoforms were identified in both HLE-B3 and MG63 cells under control conditions, in contrast to previously reported results by our team under the particularly experimental conditions (10, 13), probably due to the increased sensitivity of our new assay. Very interestingly, the IGF-1Eb isoform was never identified in both estrogen receptor (ER)-positive or -negative breast cancer cell lines MCF7 and MDA-MB-231, respectively, under our experimental conditions.

Identification of IGF-1 isoform origin; the prevalence of class-1 peptides. We next wondered about the transcription initiation sites of IGF-1 peptides located either on exon 1 or 2 using specific primers, as described, for class 1 or 2

molecules, respectively. The levels of class 1 or 2 originated peptides are shown in Figure 4B and C, although for some cell lines amplification of classes' transcripts was not possible, probably due to the extended length of some amplicons. Most isoforms are found to be mainly and/or exclusively transcribed under the control of exon1 promoter, especially those regarding the IGF1-Eb and Ec peptide. Interestingly, IGF-1Ea and IGF-1Eb isoforms were found to be strictly originated from exon 2 in HeLa and MG63 cells, respectively. Both promoters, located either to exon 1 or exon 2, were found to contribute equally to the transcription of all three isoforms in DLD-1 cells, whereas class 2 served as the predominant origin for all three peptides only in KLE cells.

E2 and dexa modulated pro-IGF1-Ea and Eb expression in PC3 cells. We have previously addressed the expression pattern of pro-IGF1-Ea, -Eb and -Ec in PC3 cells under control conditions (11). Since PC3 cells are androgen

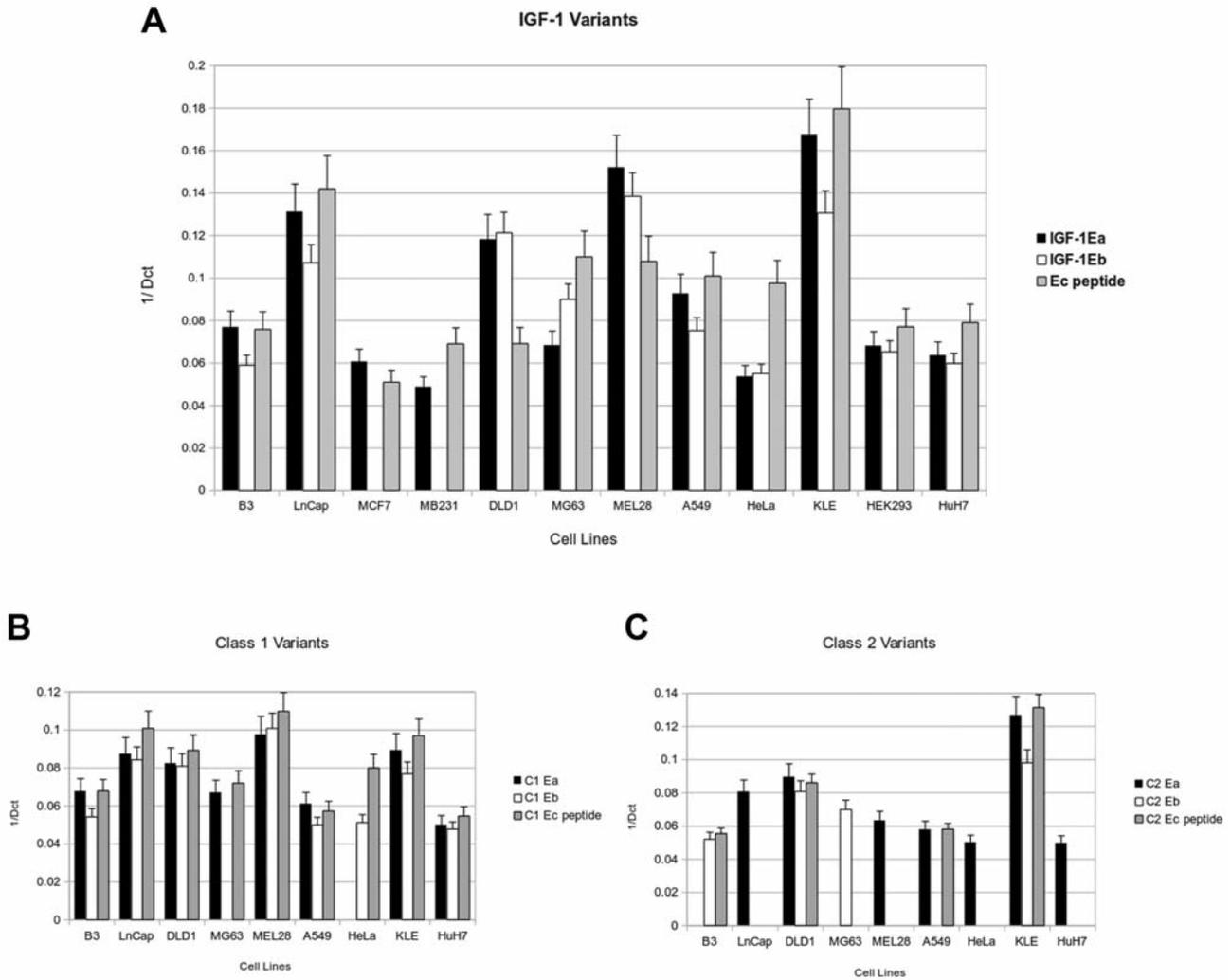


Figure 4. Levels of distinct IGF-1 transcripts expression. Because greater Ct values correspond to smaller expression, levels of relative expression were calculated after reversing the difference between the Ct of target gene and reference gene (β -actin) for each cell line (1/Dct). All samples were measured in triplicates and the values were averaged, whereas Ct values above 35 were excluded from analysis. The comparison of IGF-1 variants' expression between distinct cell lines revealed that the IGF-1Ea and Eb isoforms are expressed in greater levels in KLE, MEL28, DLD1 and LnCap cells. Additionally, the Ec peptide was found at maximum levels also in KLE and LnCap, whereas MCF7 cells showed the lowest expression. Interestingly, IGF-1Eb was completely lacking, under our experimental conditions, from both ER-positive or -negative MCF7 or MB231 breast cancer cells (A). For the majority of cell lines used, we were able to determine the classes origin of IGF-1 transcripts. Class 1 proved as the predominant origin of IGF-1 transcripts for most cell lines, especially for IGF-1Eb and Ec peptide variants (B), whereas, in some cases, exon 2 served as the only transcription initiation site (C). Ct, Threshold cycle; IGF-1, insulin-like growth factor-1.

receptor-negative cells we tested the potentially hormonal regulation of IGF-1Ea, IGF-1Eb and Ec peptide expression using 10 or 100 nM E2 or Dexa for 24 or 48 h in PC3 cells. Indeed, the expression of IGF1-Ea and IGF-1Eb isoforms was significantly increased, namely Ea was up-regulated up to 10-fold and Eb up to 4-fold, compared to control, by 10 nM E2 after a 24-h exposure ($p=0.025$ and $p=0.034$, respectively), whereas 100 nM of Dexa for 48 h significantly decreased the IGF-1Ea and Eb expression ($p=0.031$ and $p=0.042$,

respectively). One hundred nM of E2 for 24 h or 10 nM of Dexa for 48 h slightly modulated Ea and Eb expression levels compared to control. Since the last 24 aa of the carboxyl (-C) terminal of IGF-1Ec isoform have been suggested to have distinct bioactive implications in prostate cancer (14), we analyzed its regulation at the mRNA level in response to several factors in PC3 cells (Figure 4B). The majority of these factors (E2 or Dexa at 10 or 100 nM for 24 or 48 h) decreased the Ec peptide in all time and dose conditions

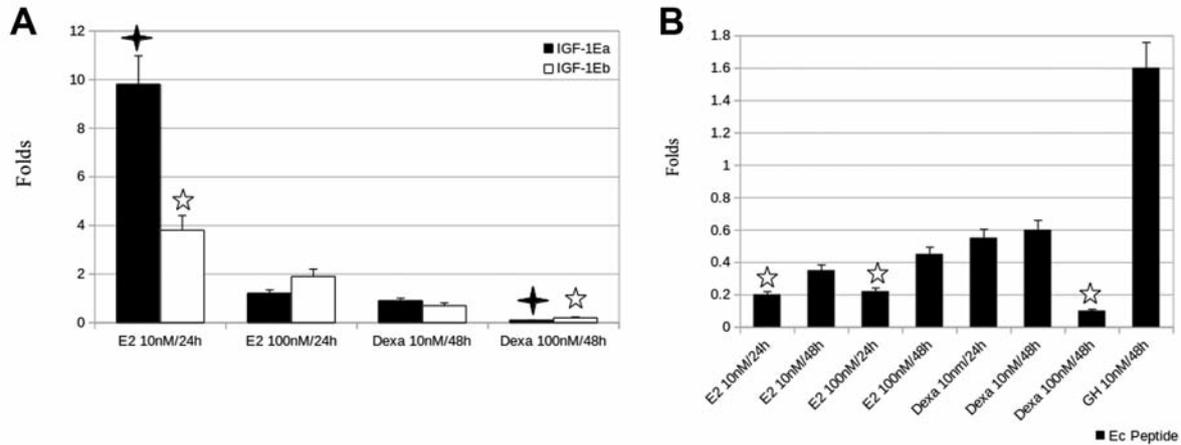


Figure 5. Hormonal regulation of IGF-1 transcripts in PC3 cells. Androgen receptor-negative prostate cancer PC3 cells were treated with 10 or 100 nM of E2, Dexa or GH for 24 or 48 h of exposure, before harvesting of cells and RNA extraction. Control cells treated with placebos for 24 or 48 h, as appropriately, and levels of each IGF-1 transcript following qRT-PCR analysis served as fold 1. Results are presented as fold changes and indicate a significant up-regulation of IGF-1Ea and IGF-1Eb isoforms by 10 nM E2 for 24 h ($p=0.025$ and $p=0.034$, respectively), whereas 100 nM of Dexa for 48 h significantly reduced both ($p=0.031$ and $p=0.042$, respectively), when compared to control (A). The Ec peptide was negatively regulated by the majority of treatment conditions used; 10 or 100 nM E2 for 24 h and 100 nM Dexa for 48 h significantly down-regulated the Ec peptide ($p=0.048$, $p=0.044$ and $p=0.032$, respectively). A small induction in Ec peptide expression was observed only after addition of 10 nM GH for 48 h (B). E2, Estradiol; Dexa, dexamethasone; GH, growth hormone; IGF-1, insulin-like growth factor-1; qRT-PCR: quantitative real-time-polymerase chain reaction.

tested. A significant reduction was observed after 10 or 100 nM E2 for 24 h ($p=0.048$ and $p=0.044$, respectively) and 100 nM Dexa after 48 h of exposure ($p=0.032$). An increase of Ec peptide expression was noticed by 10 nM of synthetic GH for 48 h, however, not a significant one. For comparison reasons and using the fold change formula, in all cases the expression level of each IGF-1 isoform in PC3 cells under control conditions served as fold 1.

Discussion

In this study we address, for the first time, the differential expression pattern of IGF-1 isoforms, including pro-, classes and E peptides, in a large number of human cancer cell lines under control and treatment conditions using a novel qPCR assay. The distinct IGF-1 transcript patterns presented herein raises the possibility for a unique role of each isoform, depending on cancer type.

It has been long enough since the scientific community started arguing about the existence and the biological activity of IGF-1 pro- and E peptides instead of the established mature IGF-1. Over the last years C-terminal E peptides have proved a major point of argument regarding their biological roles, whereas pro-IGF-1 peptides present a higher acceptance among scientists for their pathophysiological actions, probably due to the fact that they contain the mature IGF-1 sequence. Among others, our team has previously addressed unique roles for IGF-1 alternative transcripts,

especially for the Ec peptide, which notably has been suggested to have IGF-1-independent actions (7-11). Unique roles have also been addressed, by independent teams, for IGF-1Ea, IGF-1Eb and IGF-1Ec isoforms in several pathophysiological conditions, including cancer (15-24). These results, along with the data presented herein, further support the notion for distinct roles of IGF-1 alternative transcripts and implicate IGF-1 isoforms in human cancer biology, in contrast to other studies suggesting a bioactivity only for the mature IGF-1 (25, 26).

The characterization of cells lines under control conditions for IGF-1 transcripts revealed some noticeable results. First, endometrial cancer KLE cells proved to have the highest levels of IGF-1Ea and Ec peptide among all cell lines tested. Although we have previously addressed that KLE cells express all three pro-peptides (9), the direct comparison with the other cancer cells could raise the biological significance of Ea and Ec peptide in endometrial malignancy. Second, the complete lack, in our hands, of IGF-1Eb peptide in breast cancer cell lines independently of estrogen status in contrast to all other cell lines where was indeed present, could reflect a unique and probably protective effect of this variant in breast cancer progression. Additionally, estrogen-sensitive MCF7 cells were found to express the minimum levels of Ec peptide. Except cancer type and estrogen status, many other factors could also contribute for the observed IGF-1 variant expression patterns, such as the existence of viral proteins (12).

Very interestingly and for certain cell lines, we were able to determine the transcription initiation site, located either on exon 1 or 2, for the expressed IGF-1 isoforms. The importance of this finding is fortified by the limited knowledge on the potentially distinct roles of classes. We address here that class 1 transcripts are predominant in the vast majority of the cell lines used. An explanation could be the finding that class 1 transcripts may have higher transcription efficiencies, whereas class 2 transcripts could be more stable (27). Additionally, and pursuant to our results, showing a prevalence of transcripts containing exon 1, class 2 peptides were suggested to have mainly endocrine actions, whereas class 1 mostly autocrine/paracrine ones (28). Class 2 transcripts have been found to be predominant only in KLE cells. Taken together, the KLE cell line seems to present a unique pattern of IGF-1 isoforms raising, thus, the need for further studies in order to determine the importance of IGF-1 alternative transcripts in endometrial cancer pathology.

Less is also known about the response of IGF-1 variants to hormonal treatments. For these experiments we used androgen receptor-negative prostate cancer cells (PC3), where the IGF-1 isoforms expression is well-addressed (11). We found a significant regulation (up or down) of pro peptides, Ea and Eb, depending on dose and time of E2 or Dexa used. Noticeable, the Ec peptide was down-regulated almost in all hormonal conditions tested, further supporting the notion for a distinct biological significance of this molecule in prostate cancer (11, 14). We found a small induction of Ec peptide expression only after GH treatment in PC3 cells, although in normal human tissues, such as the skeletal muscle, the up-regulation at the mRNA level of all IGF-1 isoforms following GH treatment was not evident (29).

One limitation of our study is the difficulty to compare the relative expression of IGF-1 isoforms between so many cell lines, using the fold change formula due to the differential molecular background represented by each one, as well as between the isoforms themselves due to the variant efficiency of the different pairs of primers used for each transcript (Figure 3A). For that reason we used the reversed Dct formula (1/Dct) as a more suitable approach. We also failed to efficiently amplify class 1 or 2 transcripts in some cell lines probably because the length of class 1 and 2 amplicons (Table I) were above the efficiency limits of the SYBR green assay, thus, a probes' assay design could probably be proved more suitable for the distinct identification of the IGF-1 classes. The disparities of the results presented herein, for some cell lines compared to previous published results by our team, could possibly be explained by the increased sensitivity of our new assay.

To conclude, this manuscript addresses the development of a specific, sensitive and cost effective tool for the identification of almost all IGF-1 alternative transcripts. The differential expression pattern in the cell lines used, which

served as models of several human malignancies, further implicate the IGF-1 isoforms in human cancer biology. The unique pattern, which was revealed for some cancer models, such as endometrial and breast malignancies, highlight the importance of understanding the exact molecular function of the IGF-1 isoforms in these and other cancerous cases. In addition, our results may also contribute to the importance and notion for a unique and distinct role of the Ec peptide. Further studies at the mRNA and protein level, as well as determination of IGF-1 isoforms' bioactivities, are warranted to confirm our findings and define the role of these molecules in cancer.

References

- 1 Kooijman R: Regulation of apoptosis by insulin-like growth factor (IGF)-I. *Cytokine Growth Factor Rev* 17: 305-323, 2006.
- 2 Yu H and Rohan T: Role of the insulin-like growth factor family in cancer development and progression. *J Natl Cancer Inst* 92: 1472-1489, 2000.
- 3 Annunziata M, Granata R and Ghigo E: The IGF system. *Acta Diabetol* 48: 1-9, 2011.
- 4 Philippou A, Maridaki M, Pneumaticos S and Koutsilieris M: The complexity of the IGF1 gene splicing, post-translational modification and bioactivity. *Mol Med Camb Mass* 20: 202-214, 2014.
- 5 Rotwein P, Pollock KM, Didier DK and Krivi GG: Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. *J Biol Chem* 261: 4828-4832, 1986.
- 6 Chew SL, Lavender P, Clark AJ and Ross RJ: An alternatively spliced human insulin-like growth factor-I transcript with hepatic tissue expression that diverts away from the mitogenic IBE1 peptide. *Endocrinology* 136: 1939-1944, 1995.
- 7 Stavropoulou A, Halapas A, Sourla A, Philippou A, Papageorgiou E, Papalois A and Koutsilieris M: IGF-1 expression in infarcted myocardium and MGF E peptide actions in rat cardiomyocytes *in vitro*. *Mol Med* 15: 127, 2009.
- 8 Philippou A, Papageorgiou E, Bogdanis G, Halapas A, Sourla A, Maridaki M, Pissimissis N and Koutsilieris M: Expression of IGF-1 isoforms after exercise-induced muscle damage in humans: characterization of the MGF E peptide actions *in vitro*. *Vivo Athens Greece* 23: 567-575, 2009.
- 9 Milingos DS, Philippou A, Armakolas A, Papageorgiou E, Sourla A, Protopapas A, Liapi A, Antsaklis A, Mastrominas M and Koutsilieris M: Insulinlike growth factor-1Ec (MGF) expression in eutopic and ectopic endometrium: characterization of the MGF E-peptide actions *in vitro*. *Mol Med* 17: 21, 2011.
- 10 Philippou A, Armakolas A, Panteleakou Z, Pissimissis N, Nezos A, Theos A, Kaparelou M, Armakolas N, Pneumaticos SG and Koutsilieris M: IGF1Ec expression in MG-63 human osteoblast-like osteosarcoma cells. *Anticancer Res* 31: 4259-4265, 2011.
- 11 Armakolas A, Philippou A, Panteleakou Z, Nezos A, Sourla A, Petraki C and Koutsilieris M: Preferential expression of IGF-1Ec (MGF) transcript in cancerous tissues of human prostate: Evidence for a novel and autonomous growth factor activity of MGF E peptide in human prostate cancer cells. *The Prostate* 70: 1233-1242, 2010.

- 12 Durzyńska J and Barton E: IGF expression in HPV-related and HPV-unrelated human cancer cells. *Oncol Rep* 32: 893-900, 2014.
- 13 Moschos MM, Armakolas A, Philippou A, Pissimissis N, Panteleakou Z, Nezos A, Kaparelou M and Koutsilieris M: Expression of the insulin-like growth factor 1 (IGF-1) and type I IGF receptor mRNAs in human HLE-B3 lens epithelial cells. *In Vivo* 25: 179-184, 2011.
- 14 Philippou A, Armakolas A and Koutsilieris M: Evidence for the Possible Biological Significance of the *igf-1* Gene Alternative Splicing in Prostate Cancer. *Front Endocrinol* 4: 31, 2013.
- 15 Durzyńska J, Philippou A, Brisson BK, Nguyen-McCarty M and Barton ER: The pro-Forms of Insulin-Like Growth Factor I (IGF-I) Are Predominant in Skeletal Muscle and Alter IGF-I Receptor Activation. *Endocrinology* 154: 1215-1224, 2013.
- 16 Hede MS, Salimova E, Piszczek A, Perlas E, Winn N, Nastasi T and Rosenthal N: E-peptides control bioavailability of IGF-1. *PLoS One* 7: e51152, 2012.
- 17 Vinciguerra M, Santini MP, Claycomb WC, Ladurner AG and Rosenthal N: Local IGF-1 isoform protects cardiomyocytes from hypertrophic and oxidative stresses *via* SirT1 activity. *Aging* 2: 43, 2010.
- 18 Poudel B, Bilbao D, Sarathchandra P, Germack R, Rosenthal N and Santini MP: Increased cardiogenesis in P19-GFP teratocarcinoma cells expressing the propeptide IGF-1Ea. *Biochem Biophys Res Commun* 416: 293-299, 2011.
- 19 Brisson BK, Spinazzola J, Park S and Barton ER: Viral expression of insulin-like growth factor I E-peptides increases skeletal muscle mass but at the expense of strength. *AJP Endocrinol Metab* 306: E965-E974, 2014.
- 20 Durzyńska J, Wardziński A, Koczorowska M, Goździcka-Józefiak A and Barton E: Human Eb Peptide: Not just a By-product of Pre-pro-IGF1b Processing? *Horm Metab Res* 45: 415-422, 2013.
- 21 Quesada A, Ogi J, Schultz J and Handforth A: C-terminal mechano-growth factor induces heme oxygenase-1-mediated neuroprotection of SH-SY5Y cells *via* the protein kinase C/Nrf2 pathway. *J Neurosci Res* 89: 394-405, 2011.
- 22 Cui H, Yi Q, Feng J, Yang L and Tang L: Mechano growth factor E peptide regulates migration and differentiation of bone marrow mesenchymal stem cells. *J Mol Endocrinol* 52: 111-120, 2014.
- 23 Zhang B, Luo Q, Mao X, Xu B, Yang L, Ju Y and Song G: A synthetic mechano-growth factor E peptide promotes rat tenocyte migration by lessening cell stiffness and increasing F-actin formation *via* the FAK-ERK1/2 signaling pathway. *Exp Cell Res* 322: 208-216, 2014.
- 24 Collins JM, Goldspink PH and Russell B: Migration and proliferation of human mesenchymal stem cells is stimulated by different regions of the mechano-growth factor prohormone. *J Mol Cell Cardiol* 49: 1042-1045, 2010.
- 25 Fornaro M, Hinken AC, Needle S, Hu E, Trendelenburg A-U, Mayer A, Rosenstiel A, Chang C, Meier V, Billin AN, Becherer JD, Brace AD, Evans WJ, Glass DJ and Russell AJ: Mechano-growth factor peptide, the COOH terminus of unprocessed insulin-like growth factor 1, has no apparent effect on myoblasts or primary muscle stem cells. *AJP Endocrinol Metab* 306: E150-E156, 2014.
- 26 Matheny RW and Nindl BC: Loss of IGF-IEa or IGF-IEb Impairs Myogenic Differentiation. *Endocrinology* 152: 1923-1934, 2011.
- 27 Wang Y, Price SE and Jiang H: Cloning and characterization of the bovine class 1 and class 2 insulin-like growth factor-I mRNAs. *Domest Anim Endocrinol* 25: 315-328, 2003.
- 28 O'Sullivan DC, Szeszak TA and Pell JM: Regulation of IGF-I mRNA by GH: putative functions for class 1 and 2 message. *Am J Physiol Endocrinol Metab* 283: E251-258, 2002.
- 29 Aperghis M, Velloso CP, Hameed M, Brothwood T, Bradley L, Bouloux PMG, Harridge SDR and Goldspink G: Serum IGF-I levels and IGF-I gene splicing in muscle of healthy young males receiving rhGH. *Growth Horm IGF Res* 19: 61-67, 2009.

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